Promising Use of Cyclodextrin-Based Non-Viral Vectors for Gene and Oligonucleotide Drugs

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Abstract

Genes, short-hairpin RNA (shRNA), small-interfering RNA (siRNA), and decoy DNA can be principally used as tools for the treatment and prevention of many disorders, including but not limited to cancers, genetic disorders, and inherited diseases. This is accomplished by introducing exogenous nucleic acids into mammalian cells to modulate gene expression. However, direct use of such oligonucleotide drugs is hampered by several barriers, including their degradation by nucleases present in the blood and extracellular fluid, cell-membrane impermeability, and their retention in endosomes. To address this issue, the development of safe and effective delivery vectors has emerged as the main fundamental challenge for successful gene and oligonucleotide therapy. Due to the intrinsic risks associated with viral vectors, non-viral vectors have attracted increasing attention as gene and oligonucleotide carriers. We originally developed various cyclodextrin (CyD) conjugates with polyamidoamine (PAMAM) dendrimers as novel CyD-based polymers for the delivery of plasmid DNA, siRNA, shRNA, and decoy DNA. In this review, we describe the recent findings on PAMAM dendrimer conjugates using CyDs as carriers for gene, shRNA, siRNA, and decoy DNA delivery.

Keywords: cyclodextrin, polyamidoamine dendrimer, conjugate, DNA delivery, shRNA delivery, siRNA delivery

1. Introduction

The principle of somatic gene therapy is to introduce certain genes into selected cells to treat a genetic or acquired disease by interfering with the expression of specific proteins or potentially fixing a genetic mutation. Since the discovery of gene therapy, new perspectives for diagnosis,

prevention, and treatment of incurable diseases have evolved, including those related to various types of cancers, cardiovascular diseases, dermatological diseases, and even vision loss [1–3]. Successful gene therapy requires a delivery system capable of efficiently delivering the genetic cargo to all target cells. There are two different technologies that have been developed for this purpose: (1) viral and two types of non-viral vectors, and (2) lipofection and polyfection. As of 2012, >1800 clinical trials have been performed in >31 countries [4], with lipofection and naked DNA representing only 6.2 and 18.6% of the methods studied, respectively. However, polyfection has never been applied to such clinical trials. Notably, in July 2012, the European Medicines Agency recommended Glybera, which is a gene-therapy product based on an engineered adeno-associated viral vector for the treatment of severe lipoprotein-lipase deficiency in muscles, for approval as the first gene-therapy drug in the European Union. More recently, Strimvelis was approved by the European Commission in 2016 as the first *ex vivo* stem cell gene therapy for the treatment of patients with a very rare disorder called severe combined immunodeficiency due to adenosine deaminase deficiency. In some cases, viral vectors have the advantage of producing higher gene expression, although there are potential safety risks, such as immunogenicity that causes inflammatory reactions, toxin production, oncogenesis, and insertional mutagenesis [5]. The advantages of non-viral vectors in this context include higher safety levels, lower immunogenicity, cost-effectiveness, and their ability to attach a targeting ligand [6]. However, the efficiency of non-viral systems for gene delivery is markedly low in regard to gene transduction as compared with viral vectors, likely due to the lack of recognition receptors and lower endosomal-escape capability and nuclear-pore targeting [7]. Therefore, non-viral vectors have been continuously researched and developed in order to overcome their disadvantages and successfully achieve the desired effects of gene therapy.

The discovery of RNA interference (RNAi) as an endogenous tool capable of fine-tuning gene expression is considered as one of the most important discoveries in the recent years [8]. Since then, many efforts have been made to exploit this natural mechanism to experimentally silence target genes, as well as to study functional genomics by inhibiting gene expression via the degradation of specific mRNA. Selective gene silencing by RNAi can be achieved either by cytoplasmic delivery of double-stranded small-interfering RNA (siRNA; 21–27 bp) or by nuclear delivery of gene-expression cassettes that express short-hairpin RNA (shRNA) [9, 10].

There are many types of nucleic acid drugs, including siRNA, microRNA (miRNA), antisense oligonucleotides, decoy DNA, aptamers, and ribozymes. siRNA is a double-stranded RNA that targets specific mRNA harboring a complementary sequence and causes its degradation; whereas, miRNA is a small noncoding-RNA molecule found in plants and animals and exhibiting functions related to RNA silencing and posttranscriptional regulation of gene expression. Furthermore, antisense oligonucleotides are single-stranded DNA harboring a complementary sequence with that of RNA molecules used to inhibit its expression. Decoy DNAs are short double-stranded oligodeoxynucleotides containing the binding sequence of a transcription factor, which prevents the transcription factor from binding to the genomic promoter, thereby resulting in transcription inhibition. Additionally, an aptamer is a nucleic acid molecule (DNA or RNA) that binds to targets with high selectivity and sensitivity, whereas ribozymes are RNA enzymes capable of binding to specific RNA, resulting in sequence-specific cleavage. Considering the marketed nucleic acid drugs, we can find only

two antisense products and one aptamer product. Vitravene (fomivirsen) is the first marketed antisense product approved by the Food and Drug Administration, and represents an antiviral antisense drug used for the treatment of patients with cytomegalovirus retinitis or those with acquired immune deficiency syndrome. The second antisense product is Kynamro (mipomersen sodium), which is used to treat patients with homozygous familial hypercholesterolemia. The aptamer product is Macugen (pegaptanib sodium), which is an anti-angiogenic drug used for the treatment of age-related macular degeneration and that binds specifically to vascular endothelial growth factor protein to block its activity. However, there are currently no commercially marketed or approved decoy DNA or siRNA drugs, although several siRNA and miRNA drugs have been in clinical trials [11].

The direct use of such nucleic acid drugs has been hindered by multiple barriers that prevent these genes and nucleic acids from reaching their targets. These barriers include degradation of nucleic acids by nucleases in the blood and extracellular fluid, elimination by the reticuloendothelial system, cell-membrane impermeability, and their retention and in endosomes and subsequent degradation [12]. Consequently, the development of a delivery system capable of overcoming these barriers is extremely important to achieving the required effects of these nucleic acid drugs. Non-viral vectors have been developed for this purpose due to their higher safety levels, non-immunogenicity, and non-pathogenicity. Methods using non-viral carriers include lipofection, which depends upon liposomes to deliver the nucleic acid cargo, polyfection, which is based on the use of a polycation complex with nucleic acids, and lipopolyfection.

Polycation-based nucleic acid drug-delivery methods offer adequate efficiency in protecting the therapeutic genes against the aforementioned barriers and also help in overcoming intracellular barriers [13]. One of the most promising approaches to non-viral delivery of nucleic acids and genes involves cationic polymers due to their ease of manufacture, flexible properties, and robustness [14]. Polyamidoamine (PAMAM) Starburst dendrimers (dendrimers), which were developed by Tomalia et al., represent the first polycations exhibiting intrinsic endosomal-release activity, thereby removing the need to add external endosomolytic agents as required by other polycations, such as poly(L-lysine) [15–17]. The dendrimers are biocompatible, non-immunogenic, and water-soluble polymers possessing unique, spherical, and highly ordered structures with a narrow molecular-weight distribution, very low polydipersity, and specific size [18, 19].

These characteristics attracted many nanotechnology, pharmaceutical, and medicinal chemistry scientists interested in their use for various applications. Because, dendrimers have terminal modifiable amino-functional groups positively charged at physiological pH [20], and they are capable of forming complexes with genes [15, 21] and oligonucleotides [22] through electrostatic interactions, as well as with glycosaminoglycans on cell surfaces [23]. Furthermore, their proton-sponge effect, as well their defined shapes, explains their high degree of transfection efficiency. In this case, the proton-sponge effect is produced by the large buffering capacity of cationic polymers that stimulate endosome swelling and eventual endosomal-membrane disruption and release of nucleic acid drugs into the cytosol [24]. The ability of dendrimers to act as non-viral vectors depends greatly upon their number of generations, with the gene-transfer activity of dendrimers with a high number of generations exceeding that of those with a low number of generations, although their cytotoxicity increases as the number of generations increases [25, 26]. Consequently, there has been growing interest in
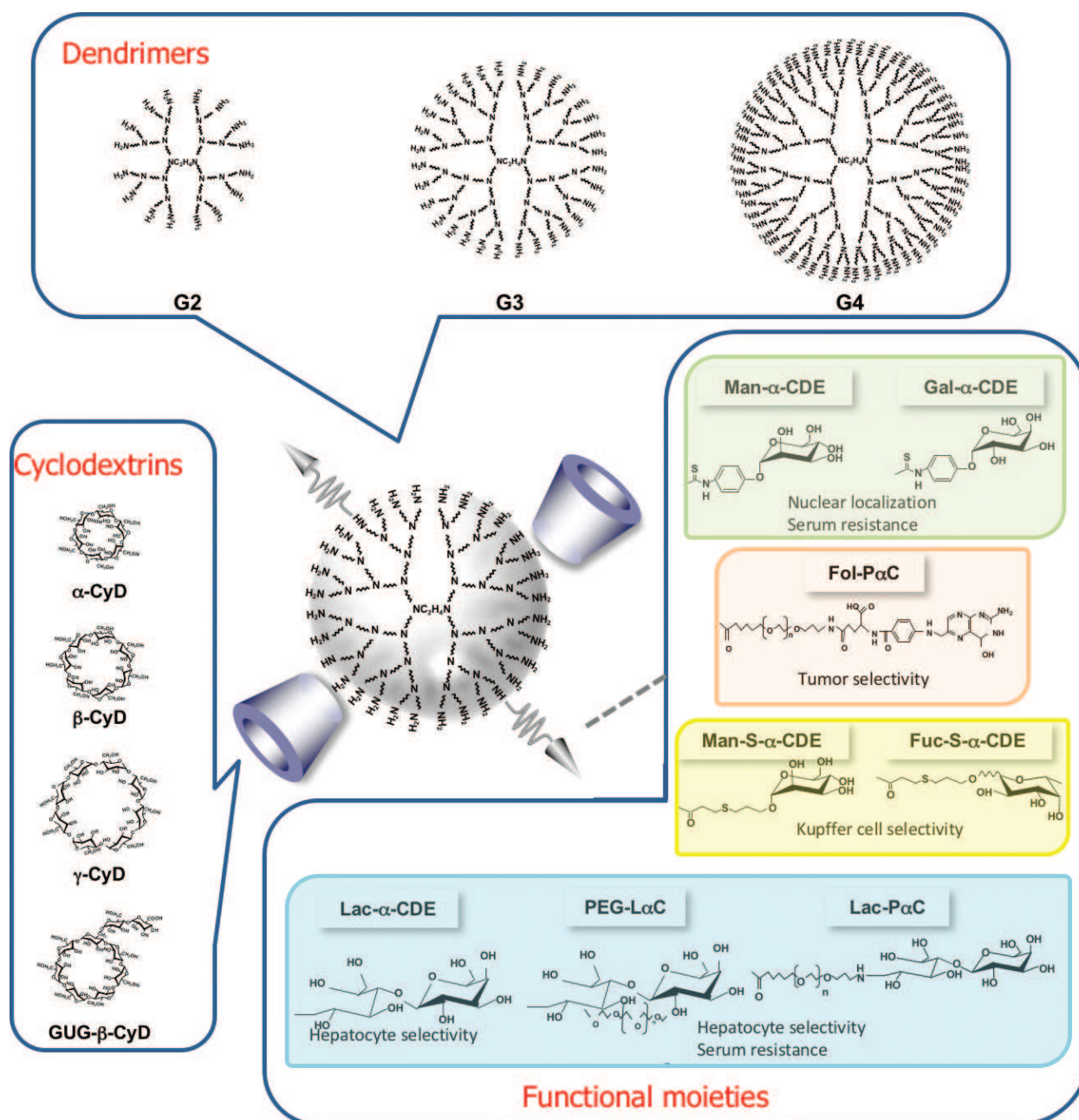
Figure 1. PAMAM dendrimer conjugates with various CyD (CDEs) and targeting ligands. GUG-β-CyD, glucuronylglucosyl-β-CyD; Man-α-CDE, mannosylated-CDE; Gal-α-CDE, galactosylated-CDE; Fol-PαC, folate-PEG-appended α-CDE; Man-S-α-CDE, mannosyloxypropylthioalkylated-α-CDE; Fuc-S-α-CDE, fucosyloxypropylthioalkylated-α-CDE; Lac-α-CDE, lactosylated-CDE; PEG-LαC, PEGylated Lac-α-CDE; Lac-PαC, lactose-PEG-appended α-CDE; PAMAM, polyamidoamine.

developing modified dendrimers possessing high levels of safety along with low numbers of generations due to their extremely low cytotoxicity [27–29].

The potential use of cyclodextrins (CyDs) as carriers of nucleic acids based of their direct interaction would not be expected, given that they exhibit very weak interactions with nucleic acids [30]. Therefore, combining CyDs with cell-penetrating nucleic acid carriers (cationic polymers) or modifying the CyD structure was necessary for their internalization. Various methods have been adopted to enhance the interactions between CyD polymers and conjugates with nucleic

acids [31]. Davis et al. modified the β-CyD structure to form a bifunctionalized β-CyD with two amine groups, allowing its incorporation into the backbone of other linear polymers [32]. The new polymer, CALAA-01, was ultimately established as the first targeted delivery mechanism of synthetic siRNA in humans for the treatment of solid tumors [33]. However, various CyD-containing polymers used as nucleic acid vectors have also been reported [34, 35]. In this regard, Arima and Motoyama originally developed various CyD conjugates with dendrimers as CyD-based polymers for the delivery of various nucleic acid drugs (**Figure 1**).

2. Polyamidoamine (PAMAM) dendrimer conjugates with α-CyDs (α-CDEs) as nucleic acid carriers

2.1. α-CDEs as plasmid (p) DNA carriers

Arima et al. prepared a variety of CyD conjugates with PAMAM dendrimers (CDE) and utilized them as gene and nucleic acid drug carriers. Originally, Arima et al. prepared dendrimer [generation 2 (G2)] conjugates with α-, β-, and γ-CyDs and named them α-, β-, and γ-CDEs (G2), respectively [36]. Among these CDEs, α-CDE (G2) exhibited the most prominent gene-transfer activity, showing 100-fold higher luciferase gene-transfer activity as compared with that of dendrimer (G2) alone or its physical mixture with α-CyD in NIH3T3 cells, a mouse embryo fibroblast cell line, and RAW264.7 cells, a mouse macrophage-like cell line. This was attributed not only to increased levels of cellular association, but also to the augmented endosomal-escape ability of the pDNA complex due to the synergy of both the proton-sponge effect and the ability of α-CyD to disrupt the endosomal membrane. Afterward, Kihara et al. examined the optimal dendrimer generation (G2, G3, or G4) and degree of substitution (DS) for α-CyD in the α-CDE molecule [37]. Consequently, α-CDE (G3, DS2) showed the highest transfection efficiency along with low cytotoxicity, which was superior to that of TransFact and Lipofectin when tested in NIH3T3 cells. Furthermore, to elucidate the reason behind the superior gene-transfer activity of α-CDE (G3, DS2), Arima et al. investigated the cellular uptake, intracellular distribution, and the physicochemical properties of pDNA complexes involving both α-CDE (G3, DS2) and the dendrimer (G3). The particle sizes, as well as the ζ-potential values, were nearly the same for both complexes. Furthermore, the enhanced gene-transfer activity could not be explained based on cellular uptake, as the values of the complexes with α-CDEs (G2, G3, and G4) were equivalent to those observed with their parent dendrimers, suggesting that factors other than cellular uptake or the physicochemical properties of the α-CDE (G3, DS2)/pDNA complexes might be strongly associated with improving gene-transfer activity. To elucidate the mechanism of cellular uptake, Arima et al. studied the effect of different endocytosis inhibitors on the cellular uptake of fluorescein isothiocyanate-labeled pDNA [(FITC)-pDNA] complexes with tetramethylrhodamine-5-(and 6)-isothiocyanate-labeled α-CDE [TRITC-α-CDE (G3)] transfection in A549 cells, ultimately using flow cytometry and confocal laser-scanning microscopy (CLSM) to observe the colocalization of TRITC-α-CDE (G3), FITC-endocytosis markers, and FITC-pDNA after transfection.

Consequently, after transfection of pDNA complexes, the complexes with TRITC-α-CDE (G3, DS2) colocalized with the endocytosis markers FITC-transferrin and FITC-cholera toxin B. Similarly, the gene-transfer activity of α-CDE (G3, DS2) was markedly lowered by the addition

of clathrin-dependent endocytosis inhibitors (i.e., chlorpromazine and sucrose) and raft-dependent endocytosis inhibitors (i.e., nystatin and filipin), but not by amiloride, the macropinocytosis inhibitor. These results suggested that the main mechanism of α-CDE (G3, DS2) uptake involved clathrin- and raft-dependent endocytosis. To confirm the release of the complex from endosomes, we observed the intracellular distribution of the α-CDE (G3, DS2)/FITC-pDNA complex by CLSM, finding that the green fluorescence originating from FITC-pDNA in the case of the α-CDE (G3, DS2) complex was predominantly localized in the cytoplasm to a much greater degree than that of the dendrimer system, confirming the improved capability for endosomal disruption conferred by the synergy between α-CDE and the proton-sponge effect of the dendrimer.

Moreover, *in vivo* studies of α-CDE (G3, DS2), as well as dendrimer complexes with pDNA, were evaluated after intravenous administration of 50 μg pDNA/mouse at a charge ratio of 10. After 12 h, organs were collected, and pDNA levels in various organs were determined. The results showed that α-CDE (G3, DS2) delivered pDNA more efficiently in the liver and kidney; however, the highest gene-expression levels were observed in the spleen. Blood-chemistry data related to α-CDE administration, including aspartate aminotransferase (AST), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), alanine aminotransferase (ALT), and creatinine (CRE) concentrations, showed minor changes as compared with those associated with the dendrimer [38]. These results suggested potential use of α-CDE (G3, DS2) as a safe and promising non-viral pDNA vector, although additional modifications of the α-CDE (G3, DS2) chemical structure were required to improve its nuclear translocation enable improved gene-expression results [39].

2.2. α-CDE (G3) as siRNA carriers

Assessment of the siRNA-carrier ability of α-CDE (G3, DS2) was performed by Tsutsumi et al. using a luciferase-reporter system [40, 41]. Their results showed significant reductions in the luciferase activity of the α-CDE (G3, DS2) system as compared with that observed in the control and accompanied by negligible cytotoxicity after transfection with the ternary complex pDNA/siRNA/α-CDE (G3, DS2). In this study, α-CDE (G3, DS2) showed superior transfection efficiency relative to that of the dendrimer and other commercially available transfection reagents. Additionally, they observed that the complex localized exclusively to the cytoplasm, where RNAi-related activity occurs, and as a result of the lack of a nuclear-translocation moiety in α-CDE (G3, DS2). Tsutsumi et al. also reported efficient knockdown of the firefly luciferase gene using a α-CDE (G3, DS2)/siRNA binary system accompanied by negligible cytotoxicity as compared with the use of siRNA complexes with commercially available transfection reagent (Lipofectamine 2000 and RNAiFect). Furthermore, the physicochemical properties, local irritation, cytotoxicity, interferon response, cellular uptake, and intracellular distribution of the siRNA complexes, as well as the RNAi activity associated with the α-CDE (G3, DS2)/siRNA complex, were evaluated on endogenous gene-expression in Colon-26-luc and NIH3T3-luc cells stably expressing the pGL3 luciferase gene. The results revealed potent RNAi activity against Lamin A/C and Fas expression along with minor cytotoxicity as compared with commercial transfection agents [40]. Additionally, siRNA complexed with α-CDE (G3, DS2) was protected from degradation by serum nucleases. Intriguingly and somewhat

similar to results observed with the α-CDE (G3, DS2)/pDNA complex, α-CDE (G3, DS2)/FITC-siRNA was delivered exclusively to the cytoplasm in NIH3T3-luc cells. Additionally, when this system was applied *in vivo* in mice bearing Colon-26-luc tumors, α-CDE (G3, DS2)/siGL3 (siRNA against pGL3 gene) showed potent RNAi activity against pGL3 expression after intravenous, as well as intratumoral, injection. Moreover, the siRNA complex neither triggered the immune response nor changed blood-chemistry data, indicating its safety. These results suggested the potential of α-CDE (G3, DS2) as a novel siRNA-carrier candidate for both *in vivo* and *in vitro* applications.

2.3. α-CDE (G3) as shRNA carriers

To improve upon and prolong the duration of RNAi-mediated gene knockdown, vector-based shRNA-expression systems were developed [42]. Upon shRNA transfection into mammalian cells, the insert containing the vector is transferred to the nucleus, integrated into the host genome, expressed, and quickly processed by Dicer-dependent cleavage and loaded into the RNA-induced silencing complex, which is then directed to the target mRNA, resulting in its degradation. A previous study demonstrated the potential of α-CDE (G3, DS2) as a novel carrier of pDNA expressing shRNA [43]. In this study, the authors used pDNA expressing shRNA against the pGL3 firefly luciferase gene (shGL3). Even at a low charge ratio, α-CDE (G3, DS2) capably formed a stable condensed complex with shGL3 and induced the conformational transition of shRNA in solution from B-form to the more compact C-form DNA. Furthermore, α-CDE (G3, DS2) markedly inhibited shGL3 degradation by DNase I, and the α-CDE (G3, DS2)/shGL3 complex showed the most potent RNAi activity at a charge ratio of 20 along with negligible cytotoxicity and without off-target effects in A549 cells, a human alveolar adenocarcinoma cell line, while also transiently expressing the luciferase gene in NIH3T3-luc cells. Moreover, the addition of sufficient amounts of siGL3 along with α-CDE (G3, DS2)/shGL3 dramatically enhanced the RNAi activity, which was ascribed to the stabilizing effect of α-CDE (G3, DS2) against DNase I degradation of the shRNA accompanied by negligible cytotoxicity. These results suggested that α-CDE (G3, DS2) possessed the potential to be a novel shRNA carrier.

2.4. Functionalized α-CDEs as cell-specific DNA and siRNA carriers

In the early 1900s, the German Noble laureate Paul Ehrlich first introduced the concept of targeted drug delivery [44, 45]. At that time, he called it the "magic bullet", as it was able to deliver the drug specifically to microbes (such as bacteria) without harming the body. His continuous research eventually led to the development of the first effective drug against syphilis (Salvarsan). The primary aim of targeted drug-delivery systems is to increase the concentration of the medication in specific areas of the body relative to others, thereby improving its therapeutic index and reducing cytotoxicity. Various approaches have been adopted to target medications to the disease site [46, 47] with many compounds internalized inside of cells via receptor-mediated endocytosis. Receptor-mediated techniques use ligands attached to polyplexes to transfect cells with selected genes. Endocytosis is then mediated by various receptors, such as asialoglycoprotein receptor (ASGPR), mannose receptor (ManR), fucose

receptor (FucR), and folate receptor (FR). However, despite the advantages offered by such systems, some drawbacks exist, including immune reactions against the carriers and rapid disposition of the carriers, as well as redistribution of the drugs after their release from the carriers [48]. Therefore, Arima et al. focused on how to improve CDEs for efficient delivery of gene and nucleic acid drugs to various organs through the attachment of various ligands to existing CDEs to aid the process of receptor-mediated endocytosis.

2.4.1. Galactosylated α-CDE as a hepatocyte-selective pDNA carrier

The liver consists mainly of hepatocytes (nearly 70%) and parenchymal cells. Gene- or drug-targeting systems designed to target the liver are usually directed to hepatocytes, which overexpress ASGPRs on their cell surface. These ASGPRs mediate the removal of potentially hazardous glycoconjugates from the blood; therefore, ASGPRs are usually targeted using galactose residues coupled with a core molecule to enhance binding [49, 50]. Many approaches, including but not limited to (1) intravenous injection of pDNA within liposomes [51, 52] or via ASGPR targeting [53] and (2) intra-portal injection of recombinant adenovirus [54] and retroviral vectors [55] have been adopted to deliver foreign genes *in vivo* to hepatocytes. Moreover, an *in vitro* system that takes advantage of ASGPR-mediated endocytosis to transfect hepatocytes with an exogenous DNA using a soluble DNA carrier was developed [56]. Various ASGPR-mediated gene-delivery systems using different polymers have also been described, including galactose-polyethylene glycol (PEG)-poly(L-lysine) [57], galactosylated PEG-graft-polyethylenimine (PEI) [58, 59], and galactosylated chitosan-grafted-PEI [60]. In order to attain hepatocyte-specificity and/or improve the efficacy of α-CDE as a gene-delivery carrier, Arima et al. attached a galactose residue to form Gal-α-CDE (G2) as a novel non-viral carrier [61]. The galactose moieties were attached to the primary amino groups of α-CDE (G2) through a spacer consisting of α-D-galctopyranosylphenyl isothiocyanate, achieving various degrees of substitutions of galactose (DSGs; 1, 4, 5, 8, and 15). Evaluation of the complexation ability of the pDNA complexes by electrophoresis showed that the Gal-α-CDEs (G2) could form complexes with pDNA; however, complexation ability decreased along with increasing DSG values, possibly due to decreases in free positive-charged primary amino groups. Moreover, the ability of these carriers to protect the pDNA from degradation by serum nucleases also decreased along with increasing DSGs, likely due to the attenuated interactions and loss of pDNA-condensation ability in the presence of high DSGs in the conjugates. Additionally, they observed that Gal-α-CDEs (G2, DSG4) elicited the most prominent gene-transfer activity relative to that of the dendrimer (G2), and α-CDE (G2, DS2) in HepG2 cells, a human hepatoma cell line, NIH3T3 cells, and A549 cells showed no cytotoxicity up to a charge ratio of 200. Surprisingly, this was independent of ASGPR expression, possibly due to the inability of the spacer to properly present the galactose residue for receptor recognition. Therefore, these results suggested the potential of Gal-α-CDE (G2, DSG4) as a novel non-viral vector independent of cell-surface ASGPR expression. It is worth mentioning that in this study, the authors used a cancer-cell line (HepG2) and not normal hepatocytes, given that they both express ASGPR at similar levels, and HepG2 cells are widely used by scientists engaged in targeting studies for genes using non-viral carriers. Surprisingly, the addition of 10% fetal bovine serum (FBS) did not alter the gene transfer activity of Gal-α-CDE (G2, DSG4); however, this activity on the part of the dendrimer (G2), as well as that of α-CDE (G2, DSG2),

as pDNA vectors was decreased by the addition of FBS. Additionally, after co-incubation of Gal-α-CDE (G2, DSG4), dendrimer (G2), or Gal-α-CDE (G2, DS2) pDNA complex with asialofetuin and galactose, only a slight decrease in gene-transfer activity was observed in HepG2 cells, with no competitive effects. Consequently, these latter results confirmed that the mechanism associated with the enhanced gene-transfer activity of Gal-α-CDE (G2, DSG4) was not ASGPR-specific, but rather possibly due to other factors, such as increased stability of the pDNA complex or changes in intracellular trafficking. Collectively, these results suggested that Gal-α-CDE (G2, DSG4) exhibited enhanced pDNA-transport activity along with low cytotoxicity and considerable resistance to serum-associated degradation and could, therefore, represent an excellent non-viral gene-delivery carrier.

2.4.2. Lactosylated α-CDEs as hepatocyte-selective pDNA and siRNA carriers

As previously mentioned, Gal-α-CDE (G2, DSG4) did not exhibit hepatocyte-specific gene-delivery activity. Therefore, Arima et al. prepared lactose-appended α-CDEs (Lac-α-CDEs) containing a glucose moiety as a spacer between the dendrimer and the lactose moiety [62, 63]. Of the various Lac-α-CDEs (G2) harboring different degrees of substitution of lactose (DSLs; 1.2, 2.6, 4.6, 6.2, and 10.2), Lac-α-CDE (G2, DSL2.6) exhibited the highest gene-transfer activity in HepG2 cells along with negligible cytotoxicity. To verify whether Lac-α-CDE (G2, DSL 2.6) could bind to galactose-binding lectins, the association constant of Lac-α-CDE (G2, DSL2.6) with peanut lectin was determined and compared with that of α-CDE (G2, DS2) using surface plasmon resonance. The results showed that the association constant of Lac-α-CDE (G2, DSL2.6) was 100-fold greater than that of α-CDE (G2, DS2). It was previously reported that the dissociation constant of asialofetuin for ASGPR located on HepG2 cells is ~3.61×10^{-9} M [64]. These results indicated that the specific galactose-binding ability of Lac-α-CDE (G2, DSL2.6) was maintained, although the magnitude was not as strong as that of asialofetuin. To confirm the ASGPR-mediated gene-transfer activity of Lac-α-CDE (G2, DSL2.6), the effects of asialofetuin, as a competitor on this activity, was examined in HepG2 cells. The results revealed that the gene-transfer activity of Lac-α-CDE (G2, DSL2.6) was significantly suppressed by asialofetuin, and that cellular association of the Lac-α-CDE (G2, DSL2.6)/Alexa-pDNA complex was markedly reduced in the presence of asialofetuin. However, no inhibitory effect of asialofetuin was observed on the activity of α-CDE (G2, DS2)/Alexa-pDNA in HepG2 cells. These results indicated that the gene-transfer activity of Lac-α-CDE (G2, DSL2.6) was mediated by ASGPR endocytosis. Arima et al. then evaluated the ability of Lac-α-CDE (G2, DSL2.6) to deliver pDNA *in vivo* using the pGL3 luciferase system in mice. The complexes were intravenously injected into mice, and after 12 h, luciferase activity was determined. They observed that luciferase activity in the Lac-α-CDE (G2, DSL2.6) system was significantly higher than that observed in the α-CDE (G2) system in the liver. Furthermore, to estimate the safety profile of Lac-α-CDE (G2, DSL2.6), the effect of its pDNA complex on blood-chemistry data, such as CRE, BUN, AST, ALT, and LDH concentrations, was analyzed following intravenous administration to mice. The ALT concentration in the Lac-α-CDE (G2, DSL2.6) system, as well as in the jetPEI-hepatocyte system, was slightly elevated (no significant difference) as compared with that observed in the control. By contrast, all other parameters in the Lac-α-CDE (G2, DSL2.6) system were almost equivalent to those of controls. These results strongly suggested that Lac-α-CDE (G2, DSL2.6) exhibited hepatocyte-specific gene-transfer activity along with a good safety profile *in vivo*.

Recently, Hayashi et al. prepared a PEGylated Lac-α-CDE [PEG-LαC (G3)] to improve *in vivo* gene-transfer activity by enhancing complex stability, as well as prolonging the half-life in circulation [62]. Of the various PEG-LαCs (G3), those with degrees of substitution of the PEG moiety (DSPs) of 2.1 [PEG-LαC (G3, DSP2.1] showed higher luciferase gene-transfer activity than other PEG-LαCs (G3, DSP4.0 and DSP6.2) in HepG2 cells along with negligible cyto-toxicity up to a charge ratio of 50. Additionally, its gene-transfer activity decreased in the presence of asialofetuin, whereas it retained significantly higher gene-transfer activity, even in the presence of 50% serum. Additionally, PEG-LαC (G3, DSP2.1) showed selective gene-transfer activity into hepatic parenchymal cells rather than hepatic non-parenchymal cells *in vivo*. Furthermore, blood-chemistry values, such as CRE, BUN, AST, ALT, and LDH concentrations, following administration of the PEG-LαC (G3, DSP2.1)/pDNA complex system were almost equivalent with those in controls, suggesting that PEG-LαC (G3, DSP2.1) showed potential as a hepatocyte-selective gene carrier both *in vitro* and *in vivo*.

The potential of PEG-LαC (G3) as a siRNA carrier was also evaluated (unpublished data), with an siRNA against transthyretin (TTR) mRNA (siTTR) used to treat TTR-related familial amyloidotic polyneuropathy (TTR-FAP). The results indicated that the PEG-LαC (G3)/siTTR complex significantly reduced TTR mRNA expression in the liver as compared with the Lac-α-CDE (G3)/siTTR complex, suggesting the potential use of PEG-LαC (G3) as a hepatocyte-selective siRNA carrier (**Figure 2**).
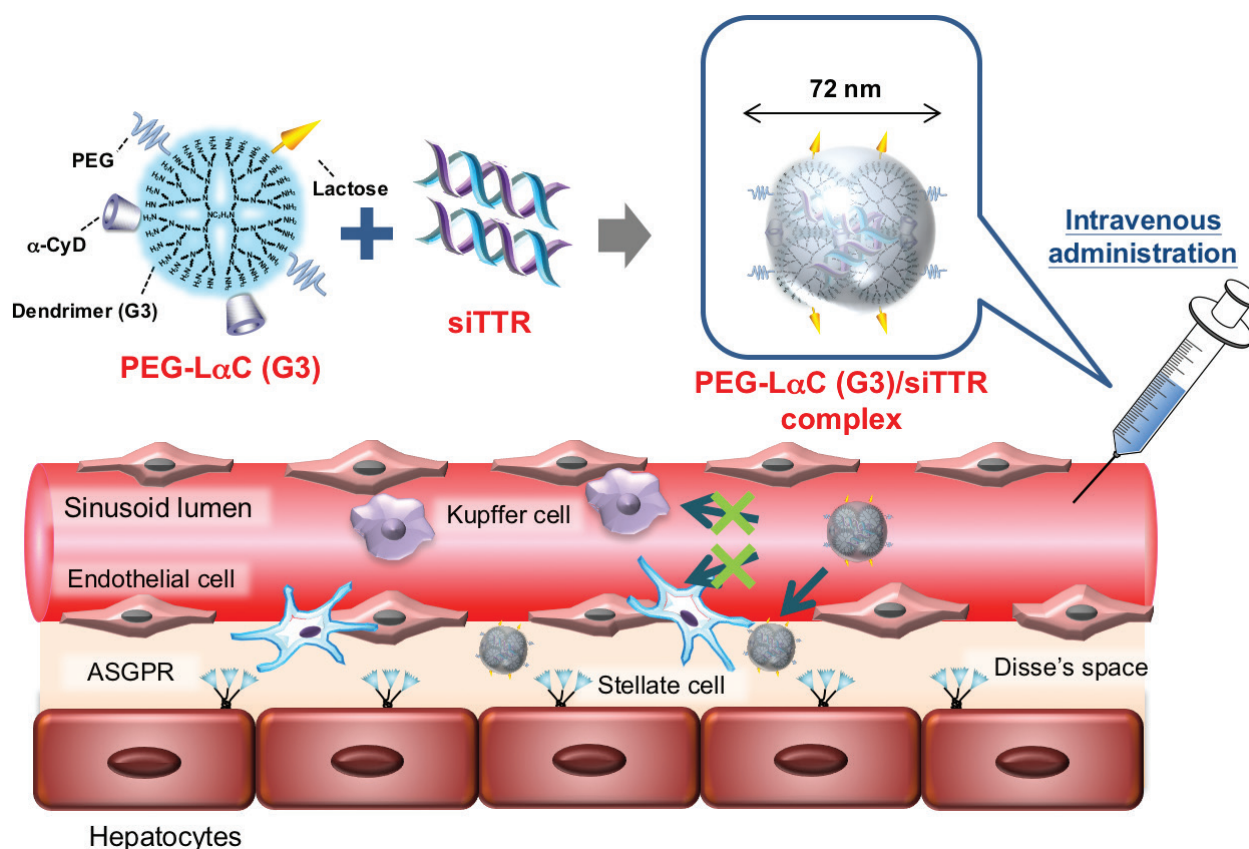

Figure 2. PEG-LαC (G3) as a targeting carrier for siTTR delivery to hepatocytes.

2.4.3. Mannose and fucose-appended α-CDEs as Kupffer cell (KC)-selective pDNA and siRNA carriers

KCs are reticuloendothelial cells that reside within the lumen of the liver sinusoid and adhere to endothelial cells that form blood-vessel walls. These non-parenchymal cells represent ~15% of the total liver cells in the human body [65] and are the first macrophages that come into contact with bacteria and bacterial toxins derived from the gastrointestinal tract [66]. KCs also play a critical role in removing harmful materials circulating in the blood. Moreover, KCs are considered an essential part of innate immunity and play an important role in the rapid response to threatening stimuli. They are also involved in the pathogenesis of different liver diseases, including viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver, development of liver fibrosis, and portal hypertension [67]. Importantly, KCs express ManR and FucR; therefore, both fucose and mannose can be used as targeting ligands on KCs used for nucleic acid delivery [68].

Arima et al. prepared mannose-appended α-CDEs (Man-α-CDE, G2) [39] to develop ManR-targeted non-viral carriers by attaching mannose residues to primary amino acid residues of α-CDE (G2) using an α-D-mannopyranosylphenyl isothiocyanate spacer. Of the various Man-α-CDEs (G2) with degrees of substitution of mannose (DSMs) of 1.1, 3.3, 4.9, and 8.3, Man-α-CDEs (G2, DSM3.3 and DSM4.9) showed higher gene-transfer activity as compared with that of dendrimer (G2) and α-CDE (G2) in NR8383 cells, a rat lung macrophage cell line, with no cytotoxicity observed up to charge ratio of 200 (carrier/pDNA). However, Man-α-CDE (G2) also showed high gene-transfer activity in A549 cells [ManR (−)], suggesting its low selectivity for ManR, possibly due to the rigidity of the spacer.

More recently, Arima et al. prepared a new Man-α-CDE with a α-D-mannopyranosylpropylthiopropionylated α-CDE (G3) spacer [Man-S-α-CDEs (G3)], which was longer and more flexible than that in Man-α-CDE [69, 70]. In this study, nuclear factor (NF)-κB was targeted due to its important role in the inflammatory response, and because it is found in almost all animal cell types. Therefore, to suppress NF-κB activation, NF-κB siRNA and NF-κB-decoy DNA were employed, with both strategies potentially attractive for the treatment of cytokine-related liver diseases, such as fulminant hepatitis. Of the various Man-S-α-CDEs with different DSMs, the NF-κB p65-specific siRNA (sip65) complex with Man-S-α-CDE (G3, DSM4) showed significantly lower *NF-κB p65* mRNA levels and nitric oxide levels in lipopolysaccharide (LPS)-stimulated NR8383 cells following ManR-mediated endocytosis (**Figure 3**). Intravenous administration of the Man-S-α-CDE (G3, DSM4)/sip65 complex increased the survival rate of the LPS-induced fulminant hepatitis mouse model via significant *in vivo* RNAi activity. These results suggested that Man-S-α-CDE (G3, DSM4) represented a potential novel KC-selective siRNA carrier.

Several reports demonstrated that NF-κB-decoy complexes harboring a liposome-functionalized fucose moiety showed higher gene-transfer efficiency as compared with mannose-appended liposomes specific for KCs [71, 72]. Therefore, Akao et al. prepared thioalkylated fucose-appended α-CDEs [Fuc-S-α-CDE (G2)] and assessed their potential as KC-selective carriers of decoy DNA (**Figure 4**) [73]. The NF-κB-decoy in complex with Fuc-S-α-CDE (G2) with an average degree of substitution of fucose (DSFuc) of two suppressed the production of

nitric oxide, as well as tumor necrosis factor-α (TNF-α) expression, in LPS-simulated NR8383 cells through FucR-mediated cellular uptake and successful endosomal escape. This complex also improved survival rates following intravenous injection into a fulminant hepatitis mouse model. Moreover, Fuc-S-α-CDE (G2, DSFuc2)/NF-κB decoy complexes showed marked accumulation in the liver relative to that observed in other organs. Furthermore, serum ALT and AST levels, as well as TNF-α levels, significantly decreased after intravenous administration of the complex in mice with fulminant hepatitis. These results suggested the potential of the Fuc-S-α-CDE (G2)/NF-κB decoy complex as an oligonucleotide therapy for fulminant hepatitis. There are many other receptors available for KC-specific drug targeting, including galactose receptors, scavenger receptors, CD36, lol-density lipoprotein receptor, and complement receptors [74]; therefore, future studies on the utility of fucose in this context should focus on its efficacy with different ligands.
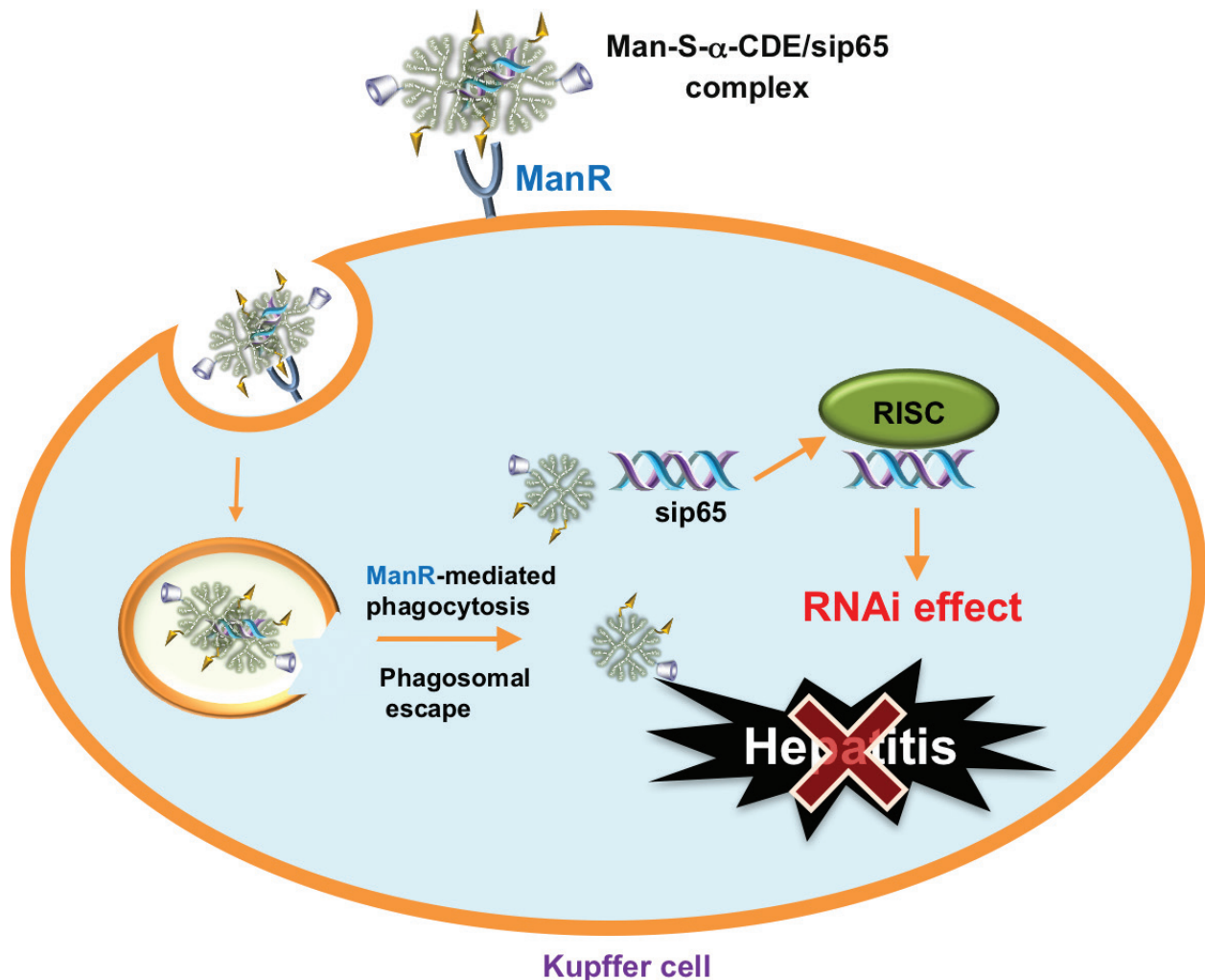

Figure 3. Man-S-α-CDE (G3) as a KC-specific targeting carrier via ManR.

2.4.4. Folate PEG-appended α-CDEs as cancer-cell-selective pDNA and siRNA carriers

To achieve maximum effective therapeutic effects against cancer using siRNAs, the design of tumor-selective delivery systems is extremely crucial. Folic acid has often been used as a tumor-specific ligand [75, 76], because it is relatively affordable as compared with other cancer-targeting ligands and is capable of high-affinity interactions with the FR-α receptor
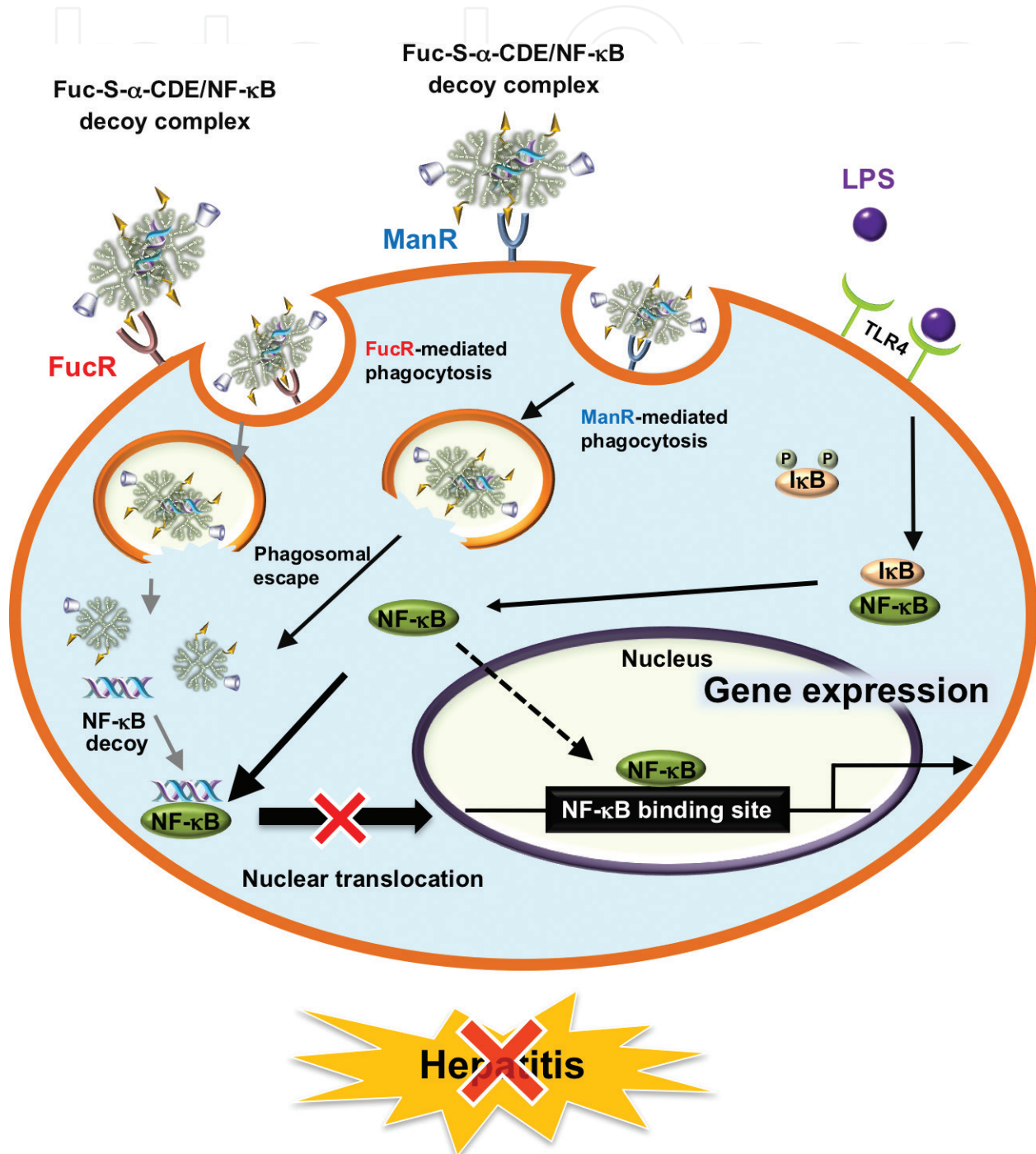

Figure 4. Fuc-S-α-CDE (G2) as targeting decoy DNA carrier to KCs.

expressed on the surface of many cancer cells ($k_d > 10^{-9}$–10^{-10} M). FR-α is highly expressed in several tumor cells, including those associated with lung, ovary, breast, kidney, and brain cancers, and is negligibly expressed in normal tissues. Additionally, as the cancer progresses in stage, the FR-α expression increases substantially. Therefore, folic acid is considered an ideal candidate cancer-cell-selective ligand.

Arima et al. prepared a folic acid-appended α-CDE (G3) with a PEG spacer [Fol-PαC (G3)] to fabricate a cancer-selective gene and siRNA carrier. Fol-PαC (G3) showed selective FR-α-overexpressing tumor-cell gene-transfer activity [77]. Specifically, Fol-PαC (G3) with an average degree of substitution of folate (DSF) of five showed significantly higher gene-transfer activity as compared with that of α-CDE (G3) in KB cells [FR-α (+)], but not in A549 [FR-α (−)] cells along with negligible cytotoxicity. Moreover, Fol-PαC (G3, DSF5) showed higher gene-transfer activity than α-CDE (G3) after intratumoral injection in mice bearing tumors.

The potential of Fol-PαC (G3) for delivery of siRNA to FR-α-overexpressing cancer cells was evaluated [78], showing that Fol-PαC (G3, DSF4) exhibited high siRNA-transfer activity in KB cells [FR-α (+)] in the absence of cytotoxicity up to a charge ratio of 100 (carrier/siRNA). Notably, the Fol-PαC (G3, DSF4)/siRNA complex showed significant RNAi activity following intratumoral injection; however, this was not the result of its dissociation in blood.

Ohyama et al. then prepared Fol-PαCs using a higher-generation dendrimer (G4) and evaluated their potential as tumor-targeting siRNA carriers *in vitro* and *in vivo* [79]. The Fol-PαC (G4, DSF2)/siRNA complex showed prominent RNAi activity based on adequate physico-chemical properties, FR-α-mediated endocytosis, efficient endosomal escape, and siRNA delivery to the cytoplasm along with negligible cytotoxicity (**Figure 5**). Most importantly, Fol-PαC (G4, DSF2) showed improved siRNA-specific blood-circulating ability, serum stability, and *in vivo* RNAi activity as compared with those observed with Fol-PαC (G3). Additionally, Fol-PαC (G4, DSF2) in complex with siRNA against Polo-like kinase 1 (siPLK1) suppressed tumor growth as compared with that observed using a control siRNA complex in mice bearing colon-26 tumor cells. These results suggested that Fol-PαC (G4) represented a potential novel tumor-targeting siRNA carrier *in vitro* and *in vivo*.

2.5. 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl (GUG)-β-CDE as a pDNA and siRNA carrier

Arima et al. clarified the importance of a spacer between the dendrimers and the targeting ligands for providing cell-specific pDNA delivery [78, 80]. However, the effect of a spacer between the CyD and the dendrimer on the gene-transfer activity of the CDE remained unknown. Consequently, a new CDE was prepared (GUG-β-CDE) utilizing a glucuronyl-glucosyl group as a spacer between the CyD and the dendrimer. Additionally, GUG-β-CyD has many advantages over the parent β-CyD, including higher water solubility, lower hemolytic activity, and increased bioadaptability [81]. Moreover, it contains a carboxyl group capable of interacting with primary amino groups present in dendrimers. Of the various GUG-β-CDEs (G2) having different DS values, GUG-β-CDE (G2, DS1.8) showed higher gene-transfer activity *in vitro* as compared with other GUG-β-CDEs (DS1.2, DS2.5, and DS4.5) [82]. Additionally, GUG-β-CDE (G2, DS1.8) showed higher gene-transfer activity relative to that of α-CDE (G2, DS1.2) and β-CDE
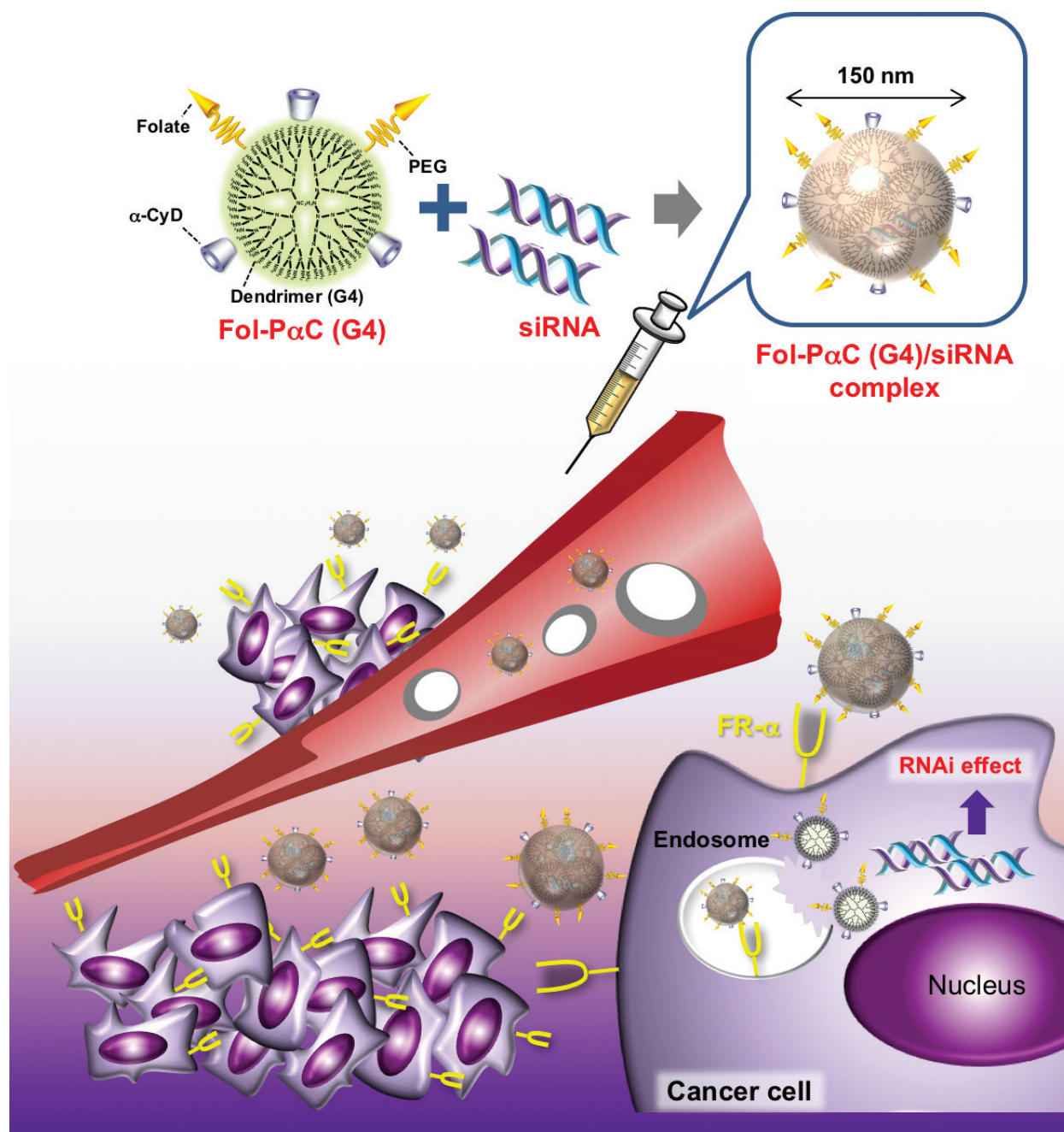
Figure 5. Fol-PαC (G4) as a targeting-siRNA carrier to folate-expressing tumor cells.

(G2, DS1.3) in A549 cells and RAW264.7 cells, respectively, possibly due to the pDNA complex exhibiting an increased ability to escape endosomes and a high degree of nuclear localization [39, 83, 84]. Moreover, *in vivo* GUG-β-CDE (G2, DS1.8) gene-transfer activity was much higher than that of α-CDE (G2, DS1.2) or β-CDE (G2, DS1.3) in kidney at 12 h after intravenous injection of the complexes in mice [85]. Therefore, GUG-β-CDE (DS1.8) might have potential as a pDNA carrier for gene-transfer targeting the kidney. Furthermore, no cytotoxicity was observed in A549 cells or RAW264.7 cells up to a charge ratio of 200 (carrier/pDNA). The hemolytic activity of GUG-β-CDE (G2, DS1.8) in rabbit red blood cells was also substantially lower than that associated with the dendrimer, and negligible changes in the blood-chemistry data were observed 12 h after

intravenous administration of the GUG-β-CDE (G2, DS1.8)/pDNA complex in mice. These results strongly suggested that this complex showed a good safety profile *in vivo* and *in vitro*, and that it might constitute an adequate carrier for gene therapy targeting kidney diseases, such as polycystic kidney disease, Alport syndrome, renal cancers, glomerulonephritis, and renal fibrosis.

Additionally, Anno et al. evaluated the potential of GUG-β-CDE (G2) as a siRNA carrier. GUG-β-CDE (G2, DS1.8) in complex with siTTR showed high RNAi activity with no cytotoxicity in HepG2 cells. Moreover, *TTR* mRNA-expression levels were reduced after intravenous administration of the complex to BALB/c mice, with only minor changes in blood-chemistry parameters, suggesting the potential of GUG-β-CDE (G2, DS1.8) as a siRNA carrier for the treatment of TTR-FAP [86].

Moreover, Ahmed et al. prepared a GUG-β-CDE using a higher-generation dendrimer (G3) [87]. Various GUG-β-CDEs (G3, DS1.6, DS3.0, DS3.7, DS5.0, and DS8.6) were prepared, with the GUG-β-CDE (G3, DS3.7)/siRNA complex showing the highest RNAi activity in KB cells transiently expressing the luciferase gene and colon 26-luc cells stably expressing the luciferase gene. Moreover, the GUG-β-CDE (G3, DS3.7)/FITC-siRNA complex showed the highest cellular uptake along with negligible cytotoxicity at a charge ratio of 20 (carrier/siRNA). Additionally, cellular uptake of the GUG-β-CDE (G3, DS3.7)/FITC-siRNA complex was significantly higher than that of the α-CDE (G3, DS2.4)/FITC-siRNA complex, suggesting GUG-β-CDE (G3, DS3.7) as a potential effective siRNA carrier. Currently, folate-PEG-appended GUG-β-CDEs (G3) are in development as a cancer-selective GUG-β-CDE (G3) variant.

2.6. Conclusion

In this review, we described various CDEs used as gene and oligonucleotide carriers. These multifunctional CDEs showed great potential as carriers for DNA and nucleic acid drugs. The advantages of these CDEs included (1) low cytotoxicity; (2) facile modification of various targeting ligands and polymers, such as PEG; and (3) enhanced endosomal-escape ability via the synergistic action of the proton-sponge effect in the dendrimer and the interaction of CyD with membrane lipids. Therefore, these CyD-based carriers have the potential for utilization as multifunctional carriers for pDNA, siRNA, decoy DNA, and shRNA.

Acknowledgements

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